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Androgen Receptor Exon 1 Mutation Causes Androgen Insensitivity by Creating Phosphorylation Site and Inhibiting Melanoma Antigen-A11 Activation of NH_2- and Carboxyl-terminal Interaction-dependent Transactivation*

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Background: Androgen receptor (AR) regulation of transcription is required for male genital development *in utero*.
Results: An AR NH_2-terminal mutation caused partial androgen insensitivity by creating a phosphorylation site and disrupting coregulator effects of melanoma antigen-A11 (MAGE-11).
Conclusion: MAGE-11 is an obligatory coregulator during human male fetal genital development.
Significance: AR signaling during the critical period of male fetal genital development requires MAGE-11.

Naturally occurring germ line mutations in the X-linked human androgen receptor (AR) gene cause incomplete masculinization of the external genitalia by disrupting AR function in males with androgen insensitivity syndrome. Almost all AR missense mutations that cause androgen insensitivity syndrome are located in the highly structured DNA and ligand binding domains. In this report we investigate the functional defect associated with an AR exon 1 missense mutation, R405S, that caused partial androgen insensitivity. The 46,XX heterozygous maternal carrier had a wild-type Arg-405 CGC allele but transmitted an AGC mutant allele coding for Ser-405. At birth, the 46,XY proband had a bifid scrotum, hypospadias, and micropenis consistent with clinical stage 3 partial androgen insensitivity. Androgen-dependent transcriptional activity of AR-R405S expressed in CV1 cells was less than wild-type AR and refractory in androgen-dependent AR NH_2- and carboxyl interaction transcription assays that depend on the coregulator effects of melanoma antigen-A11. This mutation created a Ser-405 phosphorylation site evident by the gel migration of an AR-R405S NH_2-terminal fragment as a double band that converted to the wild-type single band after treatment with λ-phosphatase. Detrimental effects of the R405S mutation were related to the proximity of the AR W*XX*LF motif ^{433}WHTLF^{437} required for melanoma antigen-A11 and p300 to stimulate transcriptional activity associated with the AR NH_2- and carboxyl-terminal interaction. We conclude that the coregulator effects of melanoma antigen-A11 on the AR NH_2- and carboxyl-terminal interaction amplify the androgen-dependent transcriptional response to p300 required for normal human male sex development *in utero*.

The androgen receptor (AR)[2] is a ligand-dependent transcription factor that mediates human male genital development *in utero* and growth at puberty. AR regulates androgen-dependent gene transcription in response to high affinity binding of testosterone or dihydrotestosterone (DHT). *In utero* human male external genital development depends on DHT, the most potent naturally occurring androgen that stabilizes the androgen-dependent AR NH_2- and carboxyl-terminal (N/C) interaction (1). Naturally occurring single amino acid missense mutations in the AR gene at Xq11–12 on the human X chromosome cause partial or complete androgen insensitivity syndrome (AIS), a disorder of sex development that results in incomplete masculinization of the 46,XY genetic male fetus. Incomplete development of external genitalia of 46,XY genetic males caused by AR gene mutations demonstrates the functional requirement for androgen-dependent AR signaling (2). Naturally occurring gene mutations in the 5α-reductase enzyme that converts testosterone to DHT can cause a phenotype similar to AIS at birth and demonstrate the requirement for optimal androgen signaling for normal human male genital development *in utero* (3).

The prevalence of AR gene mutations that cause AIS in the general population is ~0.01%. The primary diagnostic criterion for AIS is the identification of an AR gene mutation (4). CpG dinucleotides are the most prevalent mutation sites through methylation and deamination of cytidine in genomic DNA (5,

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[2] The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; N/C, NH_2- and carboxyl-terminal; AIS, androgen insensitivity syndrome; AF2, activation function 2; MAGE-11, melanoma antigen-A11; PSA, prostate-specific antigen; Luc, luciferase.

6). Most of the >200 unique naturally occurring AR gene missense mutations cause AIS by disrupting function in the highly structured DNA and ligand binding domains (7). However, the large AR NH_2-terminal region coded by exon 1 is also required for AR activity (8). AR gene missense mutations in the AR NH_2-terminal region would be expected to occur with a frequency similar to the DNA and ligand binding domains. However, most of the naturally occurring mutations in the NH_2-terminal region that cause AIS introduce premature termination codons that interrupt transcription and cause rapid degradation of the AR messenger RNA or expression of an inactive truncated protein. A notable exception is a missense mutation at the AR NH_2 terminus that interfered with translation initiation (9). The relative lack of reported missense mutations in the AR NH_2-terminal transactivation domain that cause AIS can best be explained by the unstructured nature of this region (10). Inherent structural flexibility within the AR NH_2-terminal transactivation domain may be sufficient to accommodate a missense mutation that is undetected in the general population.

AR transcriptional activity depends on interactions with coregulator proteins (11) and on the androgen-dependent interdomain AR N/C interaction between the AR NH_2-terminal F*XX*LF motif and activation function 2 (AF2) in the ligand binding domain (12, 13). The AR AF2 hydrophobic surface flanked by charged residues also binds L*XX*LL motifs of p160 coactivators but with lower affinity than AF2 binds the AR F*XX*LF motif or F*XX*LF motifs in other transcriptional regulators (1, 14, 15). The AR N/C interaction is modulated by primate-specific melanoma antigen-A11 (MAGE-11) that binds the AR F*XX*LF motif and functions synergistically with the AR N/C interaction, coregulators, and transcription factors (16–20). An AR NH_2-terminal W*XX*LF motif contributes to the AR N/C interaction through mechanisms not well understood (21, 22). Disruption of the AR N/C interaction by mutating the AR NH_2-terminal F*XX*LF motif inhibits AR transcriptional activation of androgen-responsive reporter genes (23). Although no naturally occurring mutation has been reported in the AR NH_2-terminal F*XX*LF motif, the AR N/C interaction is thought to be important for AR transcriptional activity *in vivo*. Naturally occurring mutations in the AF2 site that disrupt the AR N/C interaction and cause AIS but retain high-affinity androgen binding support a requirement for the N/C interaction in AR function (14, 24–30).

In this report, we provide evidence that the functional defect associated with naturally occurring AR NH_2-terminal missense mutation AR-R405S caused clinical stage 3 partial AIS by interference with the stimulatory effects of the AR coregulator MAGE-11. The Arg-405-to-Ser-405 mutation with amino acid numbering on the basis of human AR reported by Lubahn *et al.* (31), created a new phosphorylation site and interfered with the effects of MAGE-11 on androgen-dependent transcriptional activity associated with the AR N/C interaction. The results show that the AR NH_2-terminal ^{433}WHTLF^{437} W*XX*LF motif that lies in close proximity to the AR R405S mutation is required for transcriptional activation associated with the coregulator effects of MAGE-11 and p300.

EXPERIMENTAL PROCEDURES

Clinical Analysis—Studies were performed with informed consent and approval from the University of North Carolina at Chapel Hill and WakeMed Health and Hospitals institutional review boards. Genomic DNA sequencing of the full AR coding region performed at the Baylor College of Medicine revealed a single CGC → AGC mutation in exon 1 of the newborn 46,XY Caucasian proband. The AR exon 1 CGC → AGC mutation in the proband and mother was confirmed by sequencing genomic DNA isolated from blood leukocytes using the QIAamp Blood Maxi kit spin protocol according to the manufacturer (Qiagen). Genomic DNA (25 ng) was PCR-amplified using 2.5 units GoTaq Flexi DNA polymerase (Promega) in 50-μl reactions containing Taq Flexi buffer, 0.2 mM deoxynucleotide triphosphates (Amersham Biosciences), 2.5 mM $MgCl_2$, Q solution (Qiagen), 0.2 μM human AR primer 5′-GGGTGTGGAGGCGTTGGAGC-3′ (forward) and 0.2 μM primer 5′-TGACCCAGAACCGGGTCCCG-3′ (reverse). PCR amplification was performed at 95 °C for 5 min followed by 20 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 45 s, and then 72 °C for 7 min. The DNA sequence was determined for a 532-bp exon 1 fragment that codes for AR amino acid residues 248–424. Amino acid repeats in AR of the proband were 21 glutamine and 17 glycine residues.

DNA Vectors—pSG5-MAGE codes for full-length human MAGE-A11 (MAGE-11) (16), and pSG5-HA-p300 codes for human influenza hemagglutinin-tagged p300 (18). pCMV-AR coding for full-length human AR (31) and pCMV-AR-1–660 for the AR NH_2-terminal and DNA binding domains (8) were mutated to R405S, R405A or Y406F by QuikChange site-directed mutagenesis (Stratagene) using *PfuTurbo* DNA polymerase (Agilent). pCMV-AR-507–919 codes for the human AR DNA and ligand binding domains (8). pCMV-AR-1–503 codes for the AR NH_2-terminal domain (12), pCMV-AR-1–503-L26A,F27A for the AR F*XX*LF motif mutant, and pCMV-AR-1–503-W433A/L436A/F437A (AR-1–503-A*XX*AA) for the W*XX*LF motif mutant (21). pCMV5-AR-1–503-R405S was created by digesting pCMV5-AR-R405S with KpnI and XbaI followed by blunt end ligation. pCMV5-AR-1–503-R405A was created by digesting pNLVP16-AR-1–660-R405A with BsmI and BstEII and cloning the insert into pCMV-AR-1–503. pCMV-AR-1–660Δ406–409 was created by double PCR mutagenesis and cloning the insert into BsmI and BstEII sites in AR. The same fragment was subcloned to make pCMV-AR-1–503Δ406–409. GAL-AR-1–449 was created by PCR amplifying pCMV-AR and cloning the insert into NdeI and XhoI sites of pGALO (32, 33) and the R405S mutant by QuikChange mutagenesis. VP-AR-507–919 was created by inserting a PCR-amplified pCMV-AR fragment into the MluI and XbaI sites of pVP16 (Clontech). GAL-AR-400–449 and the A*XX*AA mutation of the W*XX*LF motif were created by subcloning PCR-amplified AR fragments into the EcoRI and BamHI sites of GALO and the R405S mutant by QuikChange mutagenesis. GAL-AR-1–422 was created by digesting GAL-AR with BstEII and XbaI and self- ligation. Luciferase reporter vectors included human prostate-specific antigen enhancer luciferase (PSA-Enh-Luc) (18, 34), prostate stem cell antigen androgen

response element-luciferase with four multimerized androgen response element-1 (PSCA-ARE-Luc) (35), p21-Luc (36) and 5XGAL4Luc3 (18, 37). Integrity of all PCR-amplified regions was verified by DNA sequencing.

Transcription Assays—Monkey kidney CV-1 cells (4×10^5/6 cm dish) were transfected with wild-type and mutant expression and luciferase reporter vector DNA using calcium phosphate precipitation. Cells were treated twice for consecutive 24-h periods with and without DHT in serum-free phenol red-free medium prior to assaying luciferase activity (19, 21). Human cervical cancer HeLa cells (5×10^4/well in 12-well plates) were transfected with expression and reporter vector DNA using FuGENE 6 (Roche). Inhibition of endogenous MAGE-11 expression in HeLa cells (1.2×10^5 cells/well in 6-well plates) was performed without antibiotics using Lipofectamine 2000 (Invitrogen) with and without 2 nM nonspecific siRNA-3 or MAGE-11 siRNA-2 and siRNA-3 (Dharmacon) (18). Luciferase activity was measured after 24 h in serum-free phenol red-free medium in the absence and presence of testosterone or DHT (1, 19) using an automated Lumistar Galaxy luminometer (BMG Labtech). Data shown (mean ± S.E.) are representative of at least three independent experiments.

Immunoblot Analysis—Monkey kidney COS-1 cells (2×10^6/10-cm dish) were transfected with 5–10 μg of plasmid DNA using DEAE dextran (1, 38). HeLa cells (7.5×10^5/10-cm dish) were transfected with 2 μg DNA using FuGENE 6 (Roche). Cells were transferred to serum-free media with and without 1 μM MG132 proteasome inhibitor (Sigma) or DHT, incubated for 24 h, and harvested in 0.1–0.2 ml of lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 50 mM NaF, 2 mM sodium vanadate, 50 mM Tris-HCl (pH 7.6), 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and complete protease inhibitor mixture (Roche). To verify phosphorylation at the R405S mutation site, 2 μl λ-phosphatase (New England Biolabs, 400,000 units/ml) was added to 40 μg of cell extract protein prepared in lysis buffer as above, except without NaF, sodium vanadate, EDTA, or deoxycholate, and incubated for 1 h at 4 °C. Extracts were analyzed on transblots of acrylamide gels containing SDS using 1 μg/ml rabbit polyclonal AR32 antipeptide antibody (39) or a 1:500 dilution of rabbit anti-GAL4 DNA binding domain antibody sc577 (Santa Cruz Biotechnology). Gels were calibrated using an EZ-Run prestained Rec protein ladder (Fisher Bioreagents).

RESULTS

Clinical Phenotype—The 46,XY Caucasian male proband was born premature at 25 weeks of gestational age with a bifid scrotum, hypospadias, micropenis, intra-abdominal testes, and absence of a uterus. Serum hormone levels (Esoterix, Inc.) obtained on the first day of life were 3252 ng/dL 17OH-pregnenolone (normal range 375–3559 ng/dL), 868 ng/dL 17OH-progesterone (124–841 ng/dL), 998 ng/dL progesterone (18–640 ng/dL), 25 ng/dL deoxycorticosterone (20–105 ng/dL), 226 ng/dL 11-desoxycortisol (18–640 ng/dL), 166 ng/dL androstenedione (63–935 ng/dL), 1.4 μg/dL cortisol (1.0–11 μg/dL), 1030 ng/dL dehydroepiandrosterone (236–3640 ng/dL), 502 ng/dL testosterone (59–125 ng/dL), 31 ng/dL DHT (10–53 ng/dL) and testosterone to DHT ratio of 16.2 (< 18).

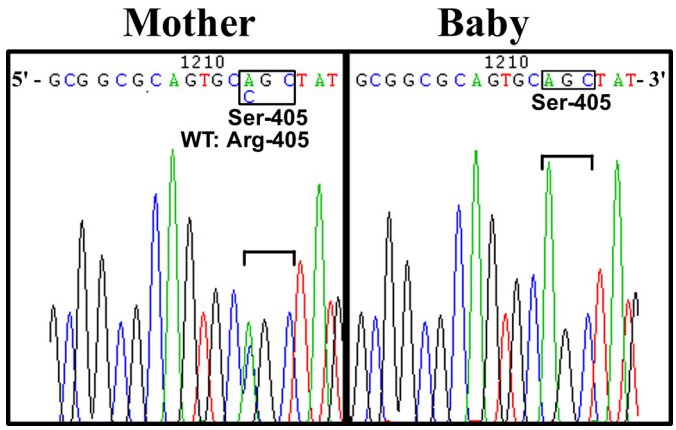

FIGURE 1. **Genomic DNA sequence from maternal heterozygous carrier and 46,XY proband with clinical stage 3 partial AIS.** Genomic DNA was isolated from blood leukocytes from the mother and newborn 46,XY proband with incomplete masculinization. DNA sequencing profiles indicate WT CGC allele coding for AR Arg-405 in the mother and AGC mutant allele coding for Ser-405 in mother and baby. The full AR coding sequence determined from baby genomic DNA revealed a single AR exon 1 CGC → AGC mutation coding for the AR R405S mutation. Thymidine nucleotide (*T*) 1210 is numbered relative to the ATG translation start site.

The incompletely masculinized external phenotype and elevated serum testosterone of the proband were consistent with clinical stage 3 partial AIS (7). Genomic DNA sequencing of the entire AR coding region indicated a single CGC → AGC mutation in exon 1 of the AR gene (Fig. 1, *right panel*). AR NH_2-terminal 21 glutamine and 17 glycine repeat lengths were in the normal range. Assays of androgen binding affinity and capacity of genital skin fibroblasts were not performed because the ligand binding domain sequence was wild-type. Although there was no family history of AIS, the maternal genomic DNA sequence in the region of the proband mutation indicated a heterozygous carrier with CGC wild-type allele coding for Arg-405 and AGC mutant allele coding for Ser-405 (Fig. 1, *left panel*), consistent with the X-linked inheritance pattern of AIS. The 46,XX maternal carrier had a normal distribution of pubic and axillary hair that may be explained by the random inactivation of one X chromosome and the partial AIS defect. The proband inherited the AGC mutant allele coding for Ser-405.

Clinical classification of stage 3 AIS and male gender assignment indicated retention of partial AR transcriptional activity. This was confirmed by a neonatal penile growth response to androgen therapy that preceded reconstructive surgery.

The R405S mutation is positioned in the region of AR NH_2-terminal activation function 1 and 27 residues from NH_2-terminal W*XX*LF motif sequence ^{433}WHTLF^{437} (Fig. 2) implicated in the androgen-dependent AR N/C interaction and transcriptional activity (13, 21–23). Expression studies using wild-type and mutant AR and select AR fragments (Fig. 2) were performed to determine the mechanism by which the R405S mutation disrupted AR transcriptional activity and caused partial AIS.

Transcriptional Activity of AR-R405S—Transcriptional consequences of the AR R405S mutation were investigated initially by determining full-length AR activation of androgen-respon-

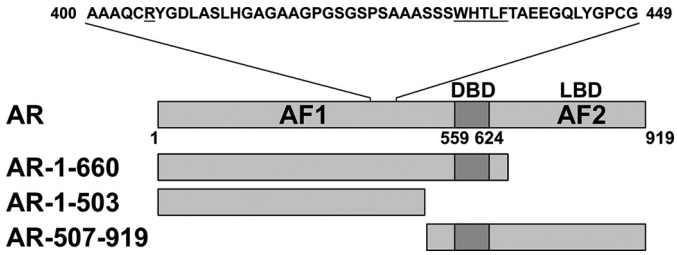

FIGURE 2. **Schematic diagram of full-length human AR and fragments.** Full-length human AR contains 919 amino acid residues on the basis of numbering of Lubahn *et al.* (31). The AR R405S mutation (wild-type Arg-405, R *underlined*) is located 27 residues from W*XX*LF motif sequence ^{433}WHTLF^{437} flanking AF1 in the NH_2-terminal region. Wild-type and mutant GAL-AR-400–449 fusion proteins, AR-1–660 NH_2-terminal and DNA binding domain (*DBD*) fragment, AR-1–503 NH_2-terminal fragment, and AR-507–919 DBD and ligand binding domain (*LBD*) fragment that contains AF2 were expressed to delineate inhibitory effects of the AR R405S mutation.

sive reporter genes. There was similar expression of human AR and AR-R405S (Fig. 3*A*). The increase in band intensity with DHT for wild-type and mutant AR suggested that the R405S mutation does not interfere with the stabilizing effect of the androgen-dependent N/C interaction between the AR NH_2-terminal F*XX*LF motif and AF2 in the ligand binding domain (21). Transcription assays using androgen-responsive reporter genes demonstrated overall reduced activity of AR-R405S compared with wild-type AR. AR-R405S transactivation of the PSA enhancer region (PSA-Enh-Luc) in CV1 cells was less than wild-type using 25 ng AR vector DNA (Fig. 3*B*). However, this difference was not seen with 50 ng of AR DNA (Fig. 3*B*). AR-R405S transcriptional activity was similar to or greater than wild-type AR in HeLa cells at increasing concentrations of testosterone or DHT with the PSA-Enh-Luc (Fig. 3*C*), prostate stem cell antigen androgen response element-Luc (Fig. 3*D*), or the p21-Luc reporter gene (Fig. 3*E*). The results indicated that the inhibitory effects of the AR R405S mutation were discernable in transcription assays performed in CV1 cells utilizing low amounts of full-length AR and androgen.

Inhibitory effects of the R405S mutation were evaluated further with respect to constitutive activity of AR NH_2-terminal and DNA binding domain fragments that lack the ligand binding domain. Expression levels of wild-type AR-1–660 and the corresponding R405S mutant were similar, and AR-1–660-R405S migrated faster than the wild-type fragment (Fig. 4*A*). Increasing amounts of AR-1–660 or AR-1–660-R405S increased transactivation of PSA-Enh-Luc to a similar extent in HeLa cells (Fig. 4*B*). Expression of MAGE-11 increased the constitutive activity of AR-1–660 and the R405S mutant (Fig. 4*C*). The R405S mutation did not diminish the synergistic increase in AR-1–660 constitutive activity by MAGE-11 and p300 (Fig. 4*D*). The R405S mutation also did not interfere with AR constitutive activity by influencing phosphorylation at Tyr-406 in a predicted Janus kinase 2 ^{406}YXXL^{409} consensus site (40). AR-1–660-Y406F and Δ406–409 expression (Fig. 4*A*) and constitutive activity (Fig. 4*B*) were like the wild type, and the MAGE-11-dependent increase in AR-1–660Δ406–409 activity was similar to AR-1–660 (Fig. 4*C*).

The results suggest that the AR R405S mutation does not interfere with transcriptional activity emanating directly from the AR NH_2-terminal transactivation region, nor did it influence phosphorylation at a potential Janus kinase 2 site. However, the results did not rule out interference of AR transcriptional activity associated with the AR N/C interaction or phosphorylation of the mutant Ser-405.

Requirement for the AR WXXLF Motif in AR N/C Interaction Transactivation by MAGE-11 and p300—The AR F*XX*LF motif binding to AF2 is the principal interaction of the androgen-dependent AR N/C interaction, whereas the AR NH_2-terminal W*XX*LF motif contributes through a mechanism that is not well understood (13). Although androgen-dependent stabilization of AR-R405S indicated an AR N/C interaction like wild-type AR (Fig. 3*A*), proximity of the R405S mutation 27 amino acid residues from the W*XX*LF motif (Fig. 2) raised the possibility that the R405S mutation might interfere with W*XX*LF motif binding to AF2 in the AR ligand binding domain or with transcriptional activity associated with the AR N/C interaction.

To test the effect of the R405S mutation on AR W*XX*LF motif binding to the AF2 region, wild-type GAL-AR-400–449 and mutants (Figs. 2 and 5*A*) were expressed in the absence and presence of DHT with VP-AR-507–919, an AR DNA and ligand binding domain fragment. It was noteworthy that GAL-AR-400–449-R405S had additional up-shifted bands not seen with GAL-AR-400–449 or the A*XX*AA mutant of the W*XX*LF motif (Fig. 5*A*, *lanes 4–6*). However, the similar androgen-dependent transcriptional activity resulting from GAL-AR-400–449 or GAL-AR-400–449-R405S interaction with VP-AR-507–919 (Fig. 5*B*) suggested that the R405S mutation does not interfere with W*XX*LF motif binding to AF2. Decreased transcriptional activity of GAL-AR-400–449-A*XX*AA expressed with VP-AR-507–919 demonstrated dependence on the W*XX*LF motif (Fig. 5*B*).

Transcriptional activity associated with the AR NH_2-terminal W*XX*LF motif region was examined in AR NH_2-terminal fragments outside the context of the AR N/C interaction. The similar increase in GAL-AR-400–449 and R405S mutant activity by MAGE-11 or p300 was not seen with the GAL-AR-400–449-A*XX*AA W*XX*LF motif mutant (Fig. 5*C*). MAGE-11 increased activity of GAL-AR-1–449 (Fig. 5*A*, *lane 2*), which contains both AR F*XX*LF and W*XX*LF motif regions, to a greater extent than it increased activity of GAL-AR-1–422 (*A*, *lane 3*), which lacks the W*XX*LF motif (*D*). However, activation by p300 was similar for GAL-AR-1–449 and GAL-AR-1–422, most likely because in the absence of the W*XX*LF motif, p300 interacts with other regions of the AR NH_2-terminal domain.

The results suggest that the AR NH_2-terminal W*XX*LF motif facilitates transcriptional activation by MAGE-11 and p300. The R405S mutation altered electrophoretic mobility of the AR-400–449 fragment but did not inhibit the N/C interaction mediated by the AR W*XX*LF motif. The principal function of the W*XX*LF motif appeared to be transcriptional activation by MAGE-11 and p300.

Phosphorylation of Mutant AR Ser-405—The altered electrophoretic migration of AR-1–660 (Fig. 4*A*) and GAL-AR-400–449 (Fig. 5*A*) by the R405S mutation suggested a change in posttranslational modification that involves phosphorylation. Phosphorylation of mutant AR Ser-405 was suggested by the double band migration of GAL-AR-400–449-R405S compared with single bands for GAL-AR-400–449 and GAL-AR-400–

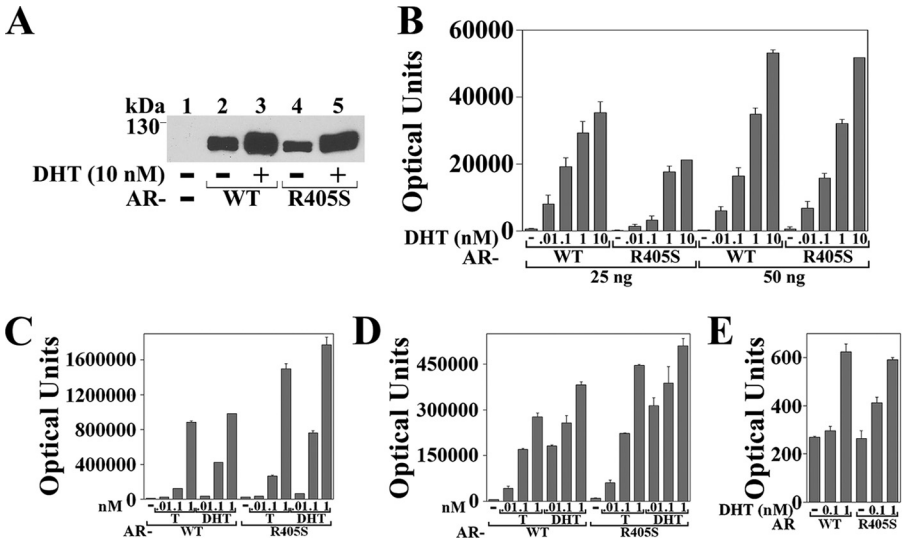

FIGURE 3. **Transcriptional activity of AR-R405S.** *A*, pCMV5 empty vector (-), pCMV-AR full-length WT, and R405S mutant (5 μg) were expressed in COS-1 cells in the absence and presence of 10 nM DHT. Cell extracts (40 μg protein/lane) were probed on the transblot using AR32 antibody. *B*, pCMV-AR WT and R405S mutant (25 and 50 ng) were expressed in CV-1 cells with 4 μg PSA-Enh-Luc. Cells were incubated with and without increasing concentrations of DHT. *C* and *D*, pCMV-AR WT and R405S mutant (10 ng) were expressed in HeLa cells with 0.25 μg PSA-Enh-Luc (*C*) or 0.25 μg PSCA-ARE-Luc (*D*). Cells were incubated with and without increasing concentrations of testosterone (*T*) or DHT. *E*, pCMV-AR WT and R405S mutant (50 ng) were expressed in HeLa cells with 0.25 μg p21-Luc. Cells were incubated with and without 0.1 or 1 nM DHT. Luciferase activity is expressed as the mean ± S.E. and is representative of at least three independent experiments.

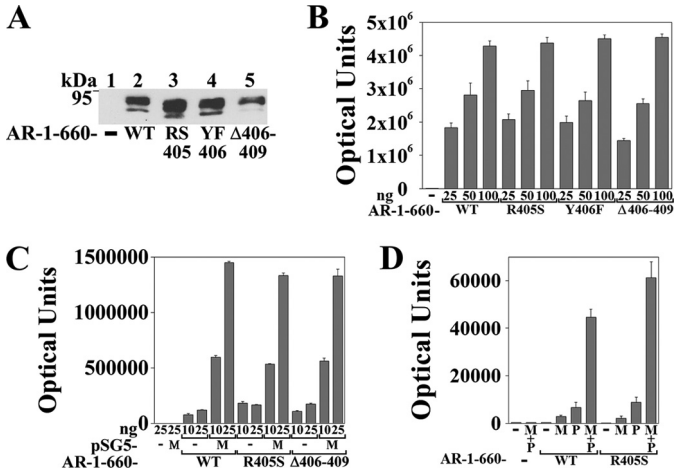

FIGURE 4. **Effect of MAGE-11 and p300 on AR-1–660 constitutive activity.** *A*, pCMV5 empty vector (-) or pCMV-AR-1–660 WT, R405S, Y406F, and Δ406–409 (8 μg DNA) were expressed in COS-1 cells. Cell extracts (50 μg protein/lane) were probed on a transblot using AR32 antibody. *B*, pCMV5 empty vector (50 ng) (-) or 25, 50, and 100 ng pCMV-AR-1–660 WT coding for AR NH_2-terminal and DNA binding domains and R405S, Y406F, and Δ406–409 mutants were expressed in HeLa cells with 0.1 μg of PSA-Enh-Luc. *C*, pCMV5 empty vector, pCMV-AR-1–660 WT, R405S, and Δ406–409 (10 ng) were expressed in HeLa cells with 0.1 μg PSA-Enh-Luc and 10 or 25 ng pSG5 empty vector (-) or pSG5-MAGE (*M*). *D*, pCMV5 empty vector (-), pCMV-AR-1–660 WT, and R405S mutant (10 ng) were expressed in CV1 cells with 2.5 μg PSA-Enh-Luc with and without 1.5 μg of pSG5 empty vector (-), 0.5 μg of pSG5-MAGE (*M*), and/or 2 μg of pSG5-HA-p300 (*P*). Luciferase activity is expressed as the mean ± S.E. and is representative of three independent experiments.

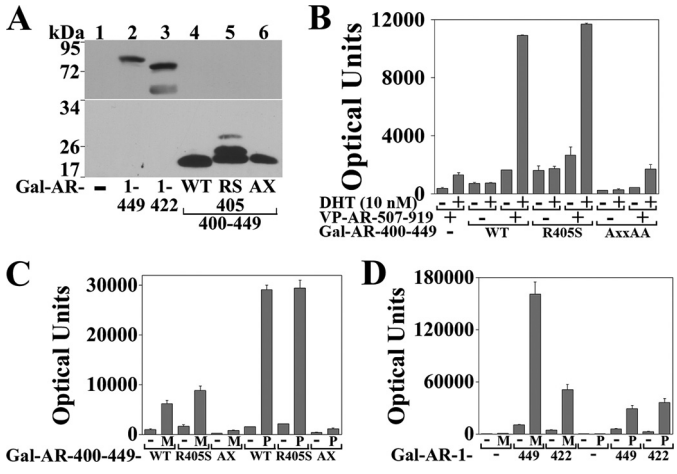

FIGURE 5. **Effect of AR R405S and W*XX*LF motif mutations on AR activation by MAGE-11 and p300.** *A*, GALO empty vector (-), GAL-AR-1–449, GAL-AR-1–422, GAL-AR-400–449 WT, R405S, and the A*XX*AA mutation of the W*XX*LF motif (10 μg) were expressed in COS-1 cells, and 60 μg of protein/lane cell extracts were analyzed on an immunoblot using GAL4 DNA binding domain antibody. *B*, GALO empty vector (-), GAL-AR-400–449 WT, R405S, and the A*XX*AA mutation of the W*XX*LF motif (50 ng) were expressed in HeLa cells with 0.1 μg 5XGAL4Luc3 and 50 ng pNLVP16 empty vector (-) or VP-AR-507–919. Cells were incubated in the absence and presence of 10 nM DHT. *C* and *D*, GAL-AR-400–449 WT, R405S, or the A*XX*AA mutation of the W*XX*LF motif (50 ng) (*C*) and 10 ng of GALO empty vector (-), GAL-AR-1–449, or GAL-AR-1–422 (*D*) were expressed in HeLa cells with 0.1 μg of 5XGAL4Luc3 with and without 100 ng of pSG5 empty vector (-), pSG5-MAGE (*M*), or pSG5-HA-p300 (*P*). Luciferase activity is expressed as the mean ± S.E. and is representative of three independent experiments.

449-A*XX*AA (Fig. 6*A*). Phosphorylation at AR Ser-405 was confirmed when the GAL-AR-400–449-R405S double band was rendered a single band like GAL-AR-400–449 after treatment with λ-phosphatase (Fig. 6*B*). A longer exposure of a transblot of GAL-AR-400–449-R405S revealed an additional up-shifted band (also evident in Fig. 5*A*) that was eliminated by λ-phosphatase (Fig. 6*C*). Treatment of cells with 10 ng/ml EGF or 10% serum did not significantly alter GAL-AR-400–449-R405S phosphorylation on the basis of intensity of the up-shifted bands (data not shown). Gel migration of AR-1–503 and the R405S and A*XX*AA mutants was also influenced by λ-phosphatase treatment (Fig. 6*D*) with varying intensities of faster migrating bands (Figs. 4*A*, 5*A*, and 6*D*), suggesting limited proteolysis.

The results suggest that loss of function by the AR R405S mutation was associated with phosphorylation of Ser-405. The

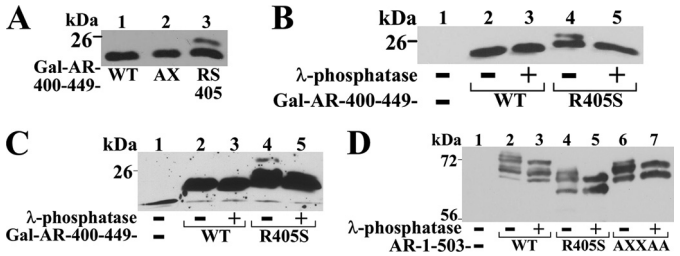

FIGURE 6. **The AR R405S mutation creates a new phosphorylation site.** *A*, GAL-AR-400–449 WT, ^{433}A*XX*AA^{437} (*AX*), and R405S mutants (10 μg) were expressed in COS-1 cells. The day before harvest, cells were incubated overnight with 1 μM MG132 proteasome inhibitor. Cell extracts (22 μg of protein/lane) were probed on the immunoblot using GAL4 DNA binding domain antibody. *B*, GALO empty vector (-), GAL-AR-400–449 WT, and R405S mutant (10 μg) were expressed in COS-1 cells. Cell extracts (40 μg of protein) were prepared in lysis buffer without phosphatase inhibitors and incubated at 4 °C for 1 h with and without λ-phosphatase as described under "Experimental Procedures." The transblot was probed with the GAL4 antibody. *C*, longer exposure of a parallel blot prepared as described in *B*. *D*, pCMV5 empty vector (-), pCMV-AR-1–503 WT, R405S, and the A*XX*AA mutations of the W*XX*LF motif (5 μg) were expressed in COS-1 cells. Cell extracts (30 μg of protein) prepared in lysis buffer without phosphatase inhibitors were treated for 1 h at 4 °C with and without λ-phosphatase and analyzed on a transblot using AR32 antibody.

AR R405S mutation was also associated with additional phosphorylation events in the AR NH_2-terminal region.

AR R405S Mutation Interferes with MAGE-11 Activation of AR N/C Interaction-dependent Transcriptional Activity—An inhibitory effect of the R405S mutation on AR activity at 0.01 nM DHT was not seen with the neutral AR-R405A mutant despite similar expression levels (Fig. 7*A*), supporting the negative influence of phosphorylation at Ser-405 on AR transcriptional activity. A detrimental effect of the R405S mutation was also seen at 1–10 pM DHT when MAGE-11 was expressed in CV1 cells (Fig. 7*B*) that have low endogenous levels of MAGE-11 (19, 41). To test for an effect on the AR N/C interaction, wild-type AR-1–503 and R405S, F*XX*LF, and W*XX*LF motif mutants that migrated as multiple bands (Fig. 7*C*) were expressed with AR-507–919 in the absence and presence of 1 nM DHT, MAGE-11 and p300. Weak androgen-dependent transcriptional activity from the intermolecular N/C interaction between AR-1–503 and AR-507–919 increased sharply with the expression of MAGE-11 or p300 (Fig. 7*D*). However, MAGE-11 did not increase transcriptional activity when AR-1–503-R405S was expressed with AR-507–919, even though activity increased with p300. Loss of MAGE-11 or p300-dependent activity when AR-1–503-L26A,F27A or AR-1–503-A*XX*AA was expressed with AR-507–919 demonstrated dependence of AR N/C interaction transcriptional activity on the AR F*XX*LF and W*XX*LF motifs.

The results suggested that MAGE-11 or p300 increases transcriptional activity associated with the AR N/C interaction and that the inhibitory effect of the AR R405S mutation on AR N/C interaction activation by MAGE-11 can be overcome by increasing the expression of p300. A requirement for endogenous p300 in transcriptional activity associated with the androgen-dependent AR N/C interaction was shown previously by silencing p300 using siRNA (18). To further demonstrate a requirement for endogenous MAGE-11, AR-1–503 was expressed with AR-507–919 in HeLa cells that have higher levels of MAGE-11 (19, 41). MAGE-11 siRNA-2, which inhibits MAGE-11 expression in HeLa cells (18, 20), inhibited transcriptional activity associated with the AR N/C interaction relative to nonspecific siRNA or MAGE-11 siRNA-3 (Fig. 7*E*) that was less effective in inhibiting MAGE-11 expression (18, 20). Inhibition of AR-R405S transcriptional activity associated with the N/C interaction by knockdown of MAGE-11 suggested residual activation of the mutant by MAGE-11.

Interference of AR transcriptional activity associated with the N/C interaction by the AR R405S mutation was investigated further in dose response studies by expressing increasing amounts of wild-type or mutant AR-1–503 (Fig. 8*A*) with AR-507–919. Androgen-dependent transcriptional activity associated with the intermolecular AR N/C interaction was similar when 10 ng of AR-1–503 or AR-1–503-R405S was expressed with AR-507–919 (Fig. 8, *B–D*). This agreed with results in Fig. 3*A* that the R405S mutation does not disrupt androgen-dependent AR stabilization by the N/C interaction. However, the dose-dependent increase in transcriptional activity seen with 25 or 50 ng of wild-type AR-1–503 did not occur with the same amounts of AR-1–503-R405S (Fig. 8*B*). This contrasted the dose-dependent increase in AR N/C interaction-dependent transcriptional activity that results from AR-1–503-Y406F or AR-1–503Δ406–919 interaction with AR-507–919 (Fig. 8*C*). Like AR-1–503-R405S, the dose-dependent increase in activity associated with the AR N/C interaction was lost when the AR-1–503-A*XX*AA mutant of the W*XX*LF motif was expressed with AR-507–919 (Fig. 8, *D* and *E*). However, there was no inhibition by AR-1–503-R405A (*E*).

Overall, the results suggest that MAGE-11 facilitates transcriptional activity associated with the AR N/C interaction that depends on the AR W*XX*LF motif and activation by p300 (Fig. 9). Inhibition of the MAGE-11-dependent increase in AR N/C interaction transcriptional activity by the AR R405S mutation was rescued by increasing the expression of p300. The data suggest that the AR R405S mutation caused partial AIS by creating a new phosphorylation site that interfered with the ability of MAGE-11 to increase AR N/C interaction-dependent transcriptional activation by p300.

DISCUSSION

Androgen Sensitivity of Normal Human Male Sex Development—Human male genital development *in utero* depends on AR signaling in response to DHT, the most potent naturally occurring androgen. The essential role of DHT in development of the human fetal male external phenotype is evident by the lack of genital development in newborns with 5α-reductase deficiency that results in insufficient conversion of testosterone to DHT (3, 42). The greater potency of DHT, which binds AR with an equilibrium binding affinity similar to testosterone (43), results from the nonpolar properties of DHT that better stabilize the androgen-dependent AR N/C interaction and slow androgen dissociation (1). This suggests that the AR N/C interaction is critical for normal human male genital development. However, no naturally occurring mutation in the AR NH_2-terminal F*XX*LF motif that mediates the AR N/C interaction has been reported. The physiological significance of the AR N/C interaction in androgen signaling has been indicated by its strict dependence on agonists with strong *in vivo*

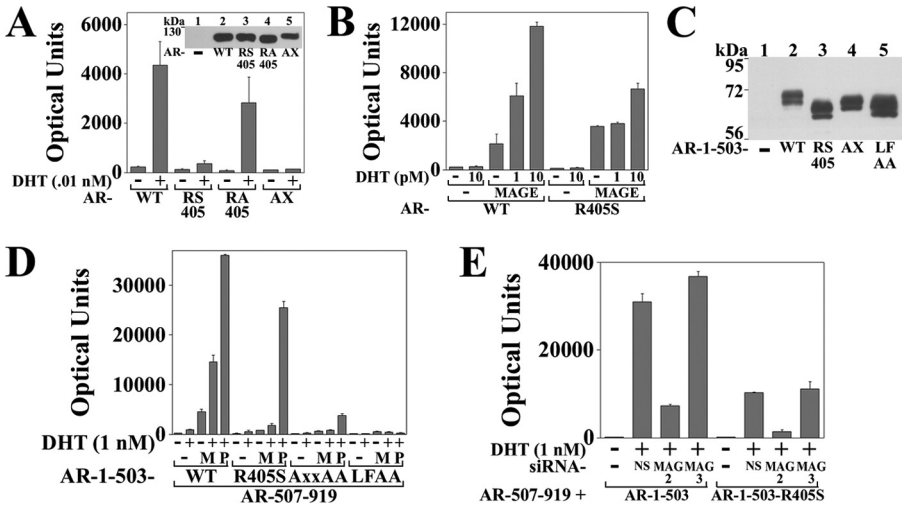

FIGURE 7. **AR R405S mutation inhibits the MAGE-11-dependent increase in AR N/C interaction-dependent transcriptional activity.** *A*, pCMV-hAR WT and R405S, R405A, and the A*XX*AA mutation of the AR W*XX*LF motif (25 ng) were expressed in CV1 cells with 2.5 μg PSA-Enh-Luc in the absence and presence of 0.01 nM DHT. *Inset*, pCMV5 empty vector (-) (5 μg) and 5 μg of pCMV-hAR WT, R405S, R405A, and the A*XX*AA mutation of the AR W*XX*LF motif were expressed in COS cells. Cell extracts (20 μg of protein/lane) were analyzed on the transblot using AR32 antibody. *B*, full-length pCMV-AR WT and the R405S mutant (25 ng) were expressed in CV1 cells with 4 μg of PSA-Enh-Luc and 0.5 μg of pSG5 empty vector (-) or pSG5-MAGE. Cells were incubated with and without 1 and 10 pM DHT. *C*, pCMV5 empty vector (-), pCMV-AR-1–503 WT, R405S, the A*XX*AA mutation of ^{433}WHTLF^{437} (*AX*), and the L26A,F27A mutation of ^{23}FQNLF^{27} (*LFAA*) (5 μg) were expressed in COS-1 cells. Cell extracts (10 μg of protein/lane) were probed on the transblot using AR32 antibody. *D*, pCMV-AR-1–503 WT (0.3 μg) and 0.3 μg of pCMV-AR-1–503-R405S, A*XX*AA, or LFAA mutants were expressed in CV1 cells with 0.1 μg of pCMV-AR-507–919 and 2.5 μg of PSA-Enh-Luc with and without 0.3 μg of pSG5 empty vector (-), 0.3 μg of pSG5-MAGE (*M*), or 1.5 μg of pSG5-HA-p300 (*P*). Cells were incubated with and without 1 nM DHT. *E*, pCMV-AR-507–919 (50 ng) was expressed in HeLa cells using Lipofectamine 2000 with 0.1 μg of PSA-Enh-Luc and 50 ng of pCMV-AR-1–503 or R405S mutant and 2 nM nonspecific (*NS*) siRNA, MAGE siRNA-2 (*MAG2*), or siRNA-3 (*MAG3*). Cells were incubated with and without 1 nM DHT, and luciferase activity was measured.

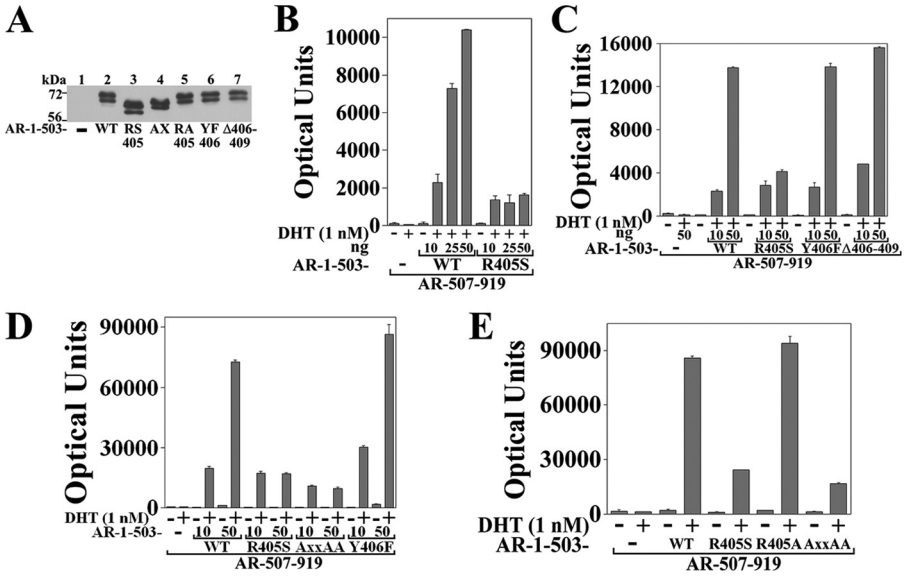

FIGURE 8. **Loss of AR N/C interaction-dependent transcriptional amplification by the AR R405S mutation.** *A*, pCMV5 empty vector (-), pCMV-AR-1–503 WT, R405S, the A*XX*AA mutation of the W*XX*LF motif (*AX*), R405A, Y406F, and Δ406–409 (5 μg) were expressed in COS-1 cells. Cell extracts (10 μg of protein/lane) were analyzed on the transblot using AR32 antibody. *B*, pCMV-AR-507–919 (50 ng) was expressed in HeLa cells with 0.1 μg of PSA-Enh-Luc and 25 ng of pCMV5 empty vector (-) or 10, 25, and 50 ng of pCMV-AR-1–503 WT or R405S mutant. *C*, pCMV-AR-507–919 (50 ng) was expressed in HeLa cells with 0.1 μg of PSA-Enh-Luc and 50 ng pCMV5 empty vector (-) or 10 and 50 ng of pCMV-AR-1–503 WT, R405S, Y406F, or Δ406–409 mutant. *D*, pCMV-AR-507–919 (50 ng) was expressed in HeLa cells with 0.1 μg of PSA-Enh-Luc and 50 ng of pCMV5 empty vector (-) or 10 and 50 ng of pCMV-AR-1–503 WT, R405S, A*XX*AA, or Y406F mutant. *E*, pCMV-AR-507–919 (50 ng) was expressed in HeLa cells with 0.1 μg of PSA-Enh-Luc and 50 ng of empty vector (-) or 50 ng of pCMV-AR-1–503 WT, R405S, R405A, or A*XX*AA mutant. *B–E*, cells were incubated with and without 1 nM DHT, and luciferase activity was measured.

androgen activity (30, 44–47) and inhibition by classical AR antagonists (12, 30). Synthetic androgens with partial agonist activity have not induced the AR N/C interaction (44, 48). Naturally occurring mutations in the AF2 site of the AR ligand binding domain that retain high affinity androgen binding can cause partial AIS by disrupting the AR N/C interaction (14, 24–30). However, the AR AF2 site also interacts with F*XX*LF motifs of coregulators and L*XX*LL motifs of p160 coactivators.

In this report, we determined the mechanism by which the naturally occurring AR NH_2-terminal germ line mutation R405S caused clinical stage 3 partial AIS in a 46,XY genetic male born with a bifid scrotum, hypospadias, micropenis, and

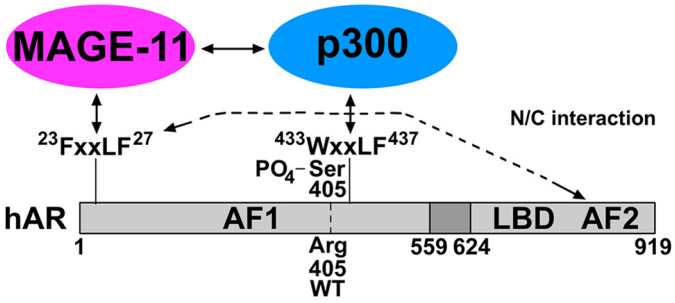

FIGURE 9. **AR R405S mutation interferes with MAGE-11 activation of AR N/C interaction-dependent transcriptional activity mediated by p300 and the AR F*XX*LF and W*XX*LF motifs.** A schematic representation is shown for the AR NH_2-terminal F*XX*LF motif binding to AF2 in the ligand binding domain (*LBD*) in the presence of DHT that results in the androgen-dependent AR N/C interaction (*dotted line*) between monomers of the AR antiparallel dimer. MAGE-11 binding to the same AR F*XX*LF motif modulates the AR N/C interaction to increase AR transcriptional activity. The AR R405S mutation caused clinical stage 3 partial androgen insensitivity syndrome by creating a new phosphorylation site and disrupting the ability of MAGE-11 to expose the W*XX*LF motif region for activation by p300. Maximal AR transactivation by MAGE-11 and p300 through AF1 and the AR NH_2-terminal F*XX*LF and W*XX*LF motif regions appears to be required for human male genital development *in utero*.

intra-abdominal testes. The data suggest that the AR R405S mutation interferes with the ability of MAGE-11, a primate-specific AR coregulator, to increase androgen-dependent transcriptional activity associated with the AR N/C interaction (Fig. 9). The coregulator function of MAGE-11 helps to recruit p300 through the AR NH_2-terminal W*XX*LF motif during the critical androgen-sensitive period of human male sex development *in utero*.

Phosphorylation in the AR NH_2-terminal Transactivation Domain—Approximately 20 different naturally occurring AR NH_2-terminal missense mutations have been reported to cause mild or partial AIS, compared with over 200 unique mutations in the AR DNA and ligand binding domains that cause partial or complete AIS. The fewer disrupting missense mutations in the AR NH_2-terminal region likely results from the absence of structure in this region that affords greater flexibility to accommodate mutations and retain function (49, 50). Similar to the AR R405S mutation, a P390S mutation also caused stage 3 partial AIS associated with micropenis at birth (51) and infertility and impaired spermatogenesis later in life (52). Similar to R405S, the P390S mutation did not inhibit full-length AR transcriptional activity when assayed using reporter genes and was postulated to alter effects of an unknown AR coactivator. The AR R405S mutation (and possibly the neighboring P390S mutation) appears to cause partial AIS by interfering with the ability of MAGE-11 to increase AR transcriptional activity associated with the AR N/C interaction mediated by p300 and the AR F*XX*LF and W*XX*LF motifs.

Both AR R405S and P390S missense mutations caused clinical stage 3 AIS by introducing a serine residue and potential new phosphorylation site. Phosphorylation is a common post-translational event in the AR NH_2-terminal region that impacts function to different extents (53–56). Some of the known AR NH_2-terminal phosphorylation sites include Ser-81, 308, and 515 phosphorylated by cyclin-dependent kinases (53, 54, 57–62), Ser-282 and 293 phosphorylated by Aurora-A kinase (63), Ser-94 (53, 54), and Ser-578 (61). None of the documented AR NH_2-terminal phosphorylation sites has been reported to be a site of a naturally occurring mutation that caused AIS. However, phosphorylation at mutant AR Ser-405 was demonstrated by altered electrophoretic migration of an AR NH_2-terminal fragment that was eliminated by treatment with λ-phosphatase. There was also evidence for additional phosphorylation events triggered by the R405S mutation, and the more neutral R405A mutation was not inhibitory. The findings suggest that detrimental effects of the R405S mutation on AR transcriptional activity were exacerbated by phosphorylation. However, it is not known to what extent phosphorylation of mutant AR Ser-405 occurred during human male genital development. Treatment of cells with EGF or serum did not alter Ser-405 phosphorylation on the basis of intensity of up-shifted bands.

Regulation of AR N/C Interaction-dependent Transcriptional Activity by MAGE-11 and p300—The AR R405S mutation lies in a short predicted α-helical region 27 residues from AR W*XX*LF motif sequence ^{433}WHTLF^{437} that functions in the AR N/C interaction (13) (Fig. 9). The W*XX*LF motif has been linked to AR transcriptional activity and is a site of somatic mutations in prostate cancer, although the mechanisms by which the W*XX*LF motif increases AR activity were unclear (13, 21, 22, 64, 65). Absence of an inhibitory effect of the R405S mutation on W*XX*LF motif binding to AF2 and on androgen-dependent AR stabilization suggested that the AR R405S mutation does not interfere with the androgen-dependent AR N/C interaction. Retention of full-length AR transcriptional activity indicated that the R405S mutation does not disrupt the AR N/C interaction as seen with mutations in the AR F*XX*LF motif (23). However, transcriptional consequences of the AR R405S mutation were evident at low DHT concentrations and in androgen-dependent AR N/C interaction assays that depended on MAGE-11.

MAGE-11 and p300 appear to be critical players in AR transcriptional activity associated with the human AR N/C interaction. siRNA studies showed that endogenous p300 (18) and MAGE-11 are required for AR N/C interaction-dependent transcriptional activity. Expression of wild-type and mutant AR NH_2-terminal fragments with the AR carboxyl-terminal fragment showed that the R405S mutation did not disrupt the AR N/C interaction but inhibited the gain in transcriptional activity seen with higher amounts of the AR NH_2-terminal fragment. This suggests that the AR R405S mutation interferes with the increase in transcriptional activity associated with the AR N/C interaction that depends on MAGE-11, p300, and the AR WXXLF motif. MAGE-11 binds the AR NH_2-terminal F*XX*LF motif that mediates the AR N/C interaction (16), interacts directly with p300 (18), and appears to be an obligatory factor in the AR transcriptional response to p300. The molecular basis for impaired male sex development in the 46,XY proband with the AR R405S mutation appears to be interference with the ability of MAGE-11 to increase AR N/C interaction-associated transcriptional activation by p300.

Most of the AR NH_2-terminal region is involved in androgen-dependent AR transcriptional activity (8). This includes an activation region between human AR amino acid residues 360 and 528 that includes the W*XX*LF motif and site of the R405S

mutation. This activation region was required for constitutive activity of AR NH_2-terminal and DNA binding domain fragments that lack the ligand binding domain but did not appear to be required for full-length AR activity (66). The AR N/C interaction may temporarily mask the W*XX*LF motif transcriptional activation surface that is exposed by MAGE-11 for enhanced transcriptional effects by p300.

Dependence on MAGE-11 for complete human male genital development *in utero* implicates a second X-linked gene as a possible cause of partial AIS. Although the human AR gene is located at Xq11–12 (2) and is wild-type in some cases of 46,XY partial AIS, the MAGE-11 gene is located at Xq28 on the human X chromosome. MAGE-11 is expressed in normal human foreskin fibroblasts and other reproductive tract tissues (16). An X-linked MAGE-11 gene mutation might account for unexplained cases of incomplete X-linked 46,XY male genital development where expression of wild-type AR is normal. However, naturally occurring mutations in the MAGE-11 gene have yet to be investigated as a cause of partial AIS.

AR mediates androgen-dependent gene transcription in all mammals, but MAGE-11 is expressed only in human and nonhuman primates (19). This suggests that MAGE-11 is not an obligatory coregulator of AR function but provides a primate-specific gain in function that augments AR signaling. AR is transcriptionally active when expressed in CV1 cells that express low levels of MAGE-11 (19, 41) and increases with increased expression of MAGE-11 (16). The functional importance of MAGE-11 as a primate-specific AR coregulator is supported by coevolution of the AR NH_2-terminal sequence with MAGE-11 expression. MAGE-11 first appeared during the primate lineage in parallel with an AR NH_2-terminal mutation from Ala-33 in less evolved mammals to Val-33 in human and nonhuman primates. Human AR Val-33 is required for the AR ^{23}FQNLF^{27} F*XX*LF motif region to interact with MAGE-11 and for competitive inhibitory effects of the AR N/C interaction on AF2 activity that are relieved by MAGE-11 binding the AR F*XX*LF motif (19). This gain in regulatory control of human AR transactivation by MAGE-11 appears to be critical for normal human male genital development *in utero*.

Clinical Prognosis—Sexual differentiation of the human male external genitalia begins at 8 to 9 weeks of gestation and is completed at ~18 weeks of gestation in response to testosterone from Leydig cells of the fetal testis stimulated by placental human chorionic gonadotropin and later by fetal pituitary luteinizing hormone (67–71). The requirement for *in utero* conversion of testosterone to DHT and the coregulator effects of MAGE-11 suggest that maximal AR signaling is necessary for normal human male fetal genital development. Androgen therapy prior to surgical repair significantly extended penile length of the newborn 46,XY proband with the AR R405S mutation and clinical stage 3 partial AIS. Partial fetal resistance to androgen that caused incomplete masculinization at birth in this clinical case may allow virilization in response to the pubertal rise in testosterone, with possibly near normal male genital growth and function. If virilization is unsatisfactory at puberty, the patient will likely respond to androgen therapy that increases circulating testosterone and DHT.

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REFERENCES

1. Askew, E. B., Gampe, R. T., Jr., Stanley, T. B., Faggart, J. L., and Wilson, E. M. (2007) Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. *J. Biol. Chem.* **282,** 25801–25816
2. Brown, C. J., Goss, S. J., Lubahn, D. B., Joseph, D. R., Wilson, E. M., French, F. S., and Willard, H. F. (1989) Androgen receptor locus on the human X chromosome: regional localization to Xq11–12 and description of a DNA polymorphism. *Am. J. Hum. Genet.* **44,** 264–269
3. Imperato-McGinley, J., Guerrero, L., Gautier, T., and Peterson, R. E. (1974) Steroid 5α-reductase deficiency in man. An inherited form of male pseudohermaphroditism. *Science* **186,** 1213–1215
4. Gottlieb, B., Beitel, L. K., and Trifiro, M. A. (2011) in *GeneReviews* (Pagon, R. A., Bird, T. D., Dolan, C. R., Stephens, K., eds) University of Washington, Seattle, WA
5. Cooper, D. N., and Youssoufian, H. (1988) The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78,** 151–155
6. Mancini, D., Singh, S., Ainsworth, P., and Rodenhiser, D. (1997) Constitutively methylated CpG dinucleotides as mutation hot spots in the retinoblastoma gene (RB1). *Am. J. Hum. Genet.* **61,** 80–87
7. Quigley, C. A., De Bellis, A., Marschke, K. B., el-Awady, M. K., Wilson, E. M., and French, F. S. (1995) Androgen receptor defects. Historical, clinical, and molecular perspectives. *Endocr. Rev.* **16,** 271–321
8. Simental, J. A., Sar, M., Lane, M. V., French, F. S., and Wilson, E. M. (1991) Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J. Biol. Chem.* **266,** 510–518
9. Choong, C. S., Quigley, C. A., French, F. S., and Wilson, E. M. (1996) A novel missense mutation in the amino-terminal domain of the human androgen receptor gene in a family with partial androgen insensitivity syndrome causes reduced efficiency of protein translation. *J. Clin. Invest.* **98,** 1423–1431
10. Reid, J., Kelly, S. M., Watt, K., Price, N. C., and McEwan, I. J. (2002) Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions. *J. Biol. Chem.* **277,** 20079–20086
11. Heemers, H. V., and Tindall, D. J. (2007) Androgen receptor (AR) coregulators. A diversity of functions converging on and regulating the AR transcriptional complex. *Endocr. Rev.* **28,** 778–808
12. Langley, E., Zhou, Z. X., and Wilson, E. M. (1995) Evidence for an antiparallel orientation of the ligand-activated human androgen receptor dimer. *J. Biol. Chem.* **270,** 29983–29990
13. He, B., Kemppainen, J. A., and Wilson, E. M. (2000) F*XX*LF and W*XX*LF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. *J. Biol. Chem.* **275,** 22986–22994
14. He, B., Kemppainen, J. A., Voegel, J. J., Gronemeyer, H., and Wilson, E. M. (1999) Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. *J. Biol. Chem.* **274,** 37219–37225
15. He, B., Gampe, R. T., Jr., Kole, A. J., Hnat, A. T., Stanley, T. B., An, G., Stewart, E. L., Kalman, R. I., Minges, J. T., and Wilson, E. M. (2004) Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. *Mol. Cell* **16,** 425–438
16. Bai, S., He, B., and Wilson, E. M. (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction. *Mol. Cell. Biol.* **25,** 1238–1257
17. Bai, S., and Wilson, E. M. (2008) Epidermal growth factor-dependent phosphorylation and ubiquitinylation of MAGE-11 regulates its interaction with the androgen receptor. *Mol. Cell. Biol.* **28,** 1947–1963
18. Askew, E. B., Bai, S., Blackwelder, A. J., and Wilson, E. M. (2010) Transcriptional synergy between melanoma antigen gene protein-A11 (MAGE-11) and p300 in androgen receptor signaling. *J. Biol. Chem.* **285,** 21824–21836
19. Liu, Q., Su, S., Blackwelder, A. J., Minges, J. T., and Wilson, E. M. (2011)

Gain in transcriptional activity by primate-specific coevolution of melanoma antigen-A11 and its interaction site in androgen receptor. *J. Biol. Chem.* **286,** 29951–29963
20. Askew, E. B., Bai, S., Hnat, A. T., Minges, J. T., and Wilson, E. M. (2009) Melanoma antigen gene protein-A11 (MAGE-11) F-box links the androgen receptor NH2-terminal transactivation domain to p160 coactivators. *J. Biol. Chem.* **284,** 34793–34808
21. He, B., Bowen, N. T., Minges, J. T., and Wilson, E. M. (2001) Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2. *J. Biol. Chem.* **276,** 42293–42301
22. Dehm, S. M., Regan, K. M., Schmidt, L. J., and Tindall, D. J. (2007) Selective role of an NH2-terminal W*XX*LF motif for aberrant androgen receptor activation in androgen depletion-independent prostate cancer cells. *Cancer Res.* **67,** 10067–10077
23. He, B., Lee, L. W., Minges, J. T., and Wilson, E. M. (2002) Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. *J. Biol. Chem.* **277,** 25631–25639
24. Langley, E., Kemppainen, J. A., and Wilson, E. M. (1998) Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. *J. Biol. Chem.* **273,** 92–101
25. Quigley, C. A., Tan, J. A., He, B., Zhou, Z. X., Mebarki, F., Morel, Y., Forest, M. G., Chatelain, P., Ritzén, E. M., French, F. S., and Wilson, E. M. (2004) Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH(2)- and carboxyl-terminal interaction. *Mech. Ageing Dev.* **125,** 683–695
26. Thompson, J., Saatcioglu, F., Jänne, O. A., and Palvimo, J. J. (2001) Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. *Mol. Endocrinol.* **15,** 923–935
27. Ghali, S. A., Gottlieb, B., Lumbroso, R., Beitel, L. K., Elhaji, Y., Wu, J., Pinsky, L., and Trifiro, M. A. (2003) The use of androgen receptor amino/carboxyl-terminal interaction assays to investigate androgen receptor gene mutations in subjects with varying degrees of androgen insensitivity. *J. Clin. Endocrinol. Metab.* **88,** 2185–2193
28. Jääskeläinen, J., Deeb, A., Schwabe, J. W., Mongan, N. P., Martin, H., and Hughes, I. A. (2006) Human androgen receptor gene ligand-binding-domain mutations leading to disrupted interaction between the N- and C-terminal domains. *J. Mol. Endocrinol.* **36,** 361–368
29. Deeb, A., Jääskeläinen, J., Dattani, M., Whitaker, H. C., Costigan, C., and Hughes, I. A. (2008) A novel mutation in the human androgen receptor suggests a regulatory role for the hinge region in amino-terminal and carboxy-terminal interactions. *J. Clin. Endocrinol. Metab.* **93,** 3691–3696
30. Wilson, E. M. (2011) Analysis of interdomain interactions of the androgen receptor. *Methods Mol. Biol.* **776,** 113–129
31. Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J., Higgs, H. N., Larson, R. E., French, F. S., and Wilson, E. M. (1988) The human androgen receptor. Complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. *Mol. Endocrinol.* **2,** 1265–1275
32. Finkel, T., Duc, J., Fearon, E. R., Dang, C. V., and Tomaselli, G. F. (1993) Detection and modulation *in vivo* of helix-loop-helix protein-protein interactions. *J. Biol. Chem.* **268,** 5–8
33. Dang, C. V., Barrett, J., Villa-Garcia, M., Resar, L. M., Kato, G. J., and Fearon, E. R. (1991) Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell. Biol.* **11,** 954–962
34. Huang, W., Shostak, Y., Tarr, P., Sawyers, C., and Carey, M. (1999) Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J. Biol. Chem.* **274,** 25756–25768
35. Jain, A., Lam, A., Vivanco, I., Carey, M. F., and Reiter, R. E. (2002) Identification of an androgen-dependent enhancer within the prostate stem cell antigen gene. *Mol. Endocrinol.* **16,** 2323–2337
36. Lu, S., Liu, M., Epner, D. E., Tsai, S. Y., and Tsai, M. J. (1999) Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter. *Mol. Endocrinol.* **13,** 376–384
37. Wagner, B. L., Norris, J. D., Knotts, T. A., Weigel, N. L., and McDonnell, D. P. (1998) The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Mol. Cell. Biol.* **18,** 1369–1378
38. He, B., Minges, J. T., Lee, L. W., and Wilson, E. M. (2002) The F*XX*LF motif mediates androgen receptor-specific interactions with coregulators. *J. Biol. Chem.* **277,** 10226–10235
39. Quarmby, V. E., Kemppainen, J. A., Sar, M., Lubahn, D. B., French, F. S., and Wilson, E. M. (1990) Expression of recombinant androgen receptor in cultured mammalian cells. *Mol. Endocrinol.* **4,** 1399–1407
40. Argetsinger, L. S., Kouadio, J. L., Steen, H., Stensballe, A., Jensen, O. N., and Carter-Su, C. (2004) Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity. *Mol. Cell. Biol.* **24,** 4955–4967
41. Karpf, A. R., Bai, S., James, S. R., Mohler, J. L., and Wilson, E. M. (2009) Increased expression of androgen receptor coregulator MAGE-11 in prostate cancer by DNA hypomethylation and cyclic AMP. *Mol. Cancer Res.* **7,** 523–535
42. Wilson, J. D., Griffin, J. E., and Russell, D. W. (1993) Steroid 5 α-reductase 2 deficiency. *Endocr. Rev.* **14,** 577–593
43. Wilson, E. M., and French, F. S. (1976) Binding properties of androgen receptors. Evidence for identical receptors in rat testis, epididymis, and prostate. *J. Biol. Chem.* **251,** 5620–5629
44. Kemppainen, J. A., Langley, E., Wong, C. I., Bobseine, K., Kelce, W. R., and Wilson, E. M. (1999) Distinguishing androgen receptor agonists and antagonists. Distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Mol. Endocrinol.* **13,** 440–454
45. He, B., and Wilson, E. M. (2002) The NH(2)-terminal and carboxyl-terminal interaction in the human androgen receptor. *Mol. Genet. Metab.* **75,** 293–298
46. Wilson, E. M. (2009) in Androgen *Action in Prostate Cancer* (Tindall, D., Mohler, J., eds) pp.241–267, Springer, New York
47. Centenera, M. M., Harris, J. M., Tilley, W. D., and Butler, L. M. (2008) The contribution of different androgen receptor domains to receptor dimerization and signaling. *Mol. Endocrinol.* **22,** 2373–2382
48. Schmidt, A., Kimmel, D. B., Bai, C., Scafonas, A., Rutledge, S., Vogel, R. L., McElwee-Witmer, S., Chen, F., Nantermet, P. V., Kasparcova, V., Leu, C. T., Zhang, H. Z., Duggan, M. E., Gentile, M. A., Hodor, P., Pennypacker, B., Masarachia, P., Opas, E. E., Adamski, S. A., Cusick, T. E., Wang, J., Mitchell, H. J., Kim, Y., Prueksaritanont, T., Perkins, J. J., Meissner, R. S., Hartman, G. D., Freedman, L. P., Harada, S., and Ray, W. J. (2010) Discovery of the selective androgen receptor modulator MK-0773 using a rational development strategy based on differential transcriptional requirements for androgenic anabolism versus reproductive physiology. *J. Biol. Chem.* **285,** 17054–17064
49. McEwan, I. J. (2012) Nuclear hormone receptors. Allosteric switches. *Mol. Cell. Endocrinol.* **348,** 345–347
50. Goglia, U., Vinanzi, C., Zuccarello, D., Malpassi, D., Ameri, P., Casu, M., Minuto, F., Foresta, C., and Ferone, D. (2011) Identification of a novel mutation in exon 1 of androgen receptor gene in an azoospermic patient with mild androgen insensitivity syndrome. Case report and literature review. *Fertil. Steril.* **96,** 1165–1169
51. Bhangoo, A., Paris, F., Philibert, P., Audran, F., Ten, S., and Sultan, C. (2010) Isolated micropenis reveals partial androgen insensitivity syndrome confirmed by molecular analysis. *Asian J. Androl.* **12,** 561–566
52. Hiort, O., Holterhus, P. M., Horter, T., Schulze, W., Kremke, B., Bals-Pratsch, M., Sinnecker, G. H., and Kruse, K. (2000) Significance of mutations in the androgen receptor gene in males with idiopathic infertility. *J. Clin. Endocrinol. Metab.* **85,** 2810–2815
53. Zhou, Z. X., Kemppainen, J. A., and Wilson, E. M. (1995) Identification of three proline-directed phosphorylation sites in the human androgen receptor. *Mol. Endocrinol.* **9,** 605–615
54. Gioeli, D., Ficarro, S. B., Kwiek, J. J., Aaronson, D., Hancock, M., Catling, A. D., White, F. M., Christian, R. E., Settlage, R. E., Shabanowitz, J., Hunt, D. F., and Weber, M. J. (2002) Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J. Biol. Chem.* **277,** 29304–29314
55. Ward, R. D., and Weigel, N. L. (2009) Steroid receptor phosphorylation. Assigning function to site-specific phosphorylation. *Biofactors* **35,** 528–536
56. Gioeli, D., and Paschal, B. M. (2012) Post-translational modification of the androgen receptor. *Mol. Cell. Endocrinol.* **352,** 70–78

57. Chen, S., Xu, Y., Yuan, X., Bubley, G. J., and Balk, S. P. (2006) Androgen receptor phosphorylation and stabilization in prostate cancer by cyclin-dependent kinase 1. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 15969–15974
58. Hsu, F. N., Chen, M. C., Chiang, M. C., Lin, E., Lee, Y. T., Huang, P. H., Lee, G. S., and Lin, H. (2011) Regulation of androgen receptor and prostate cancer growth by cyclin-dependent kinase 5. *J. Biol. Chem.* **286,** 33141–33149
59. Gordon, V., Bhadel, S., Wunderlich, W., Zhang, J., Ficarro, S. B., Mollah, S. A., Shabanowitz, J., Hunt, D. F., Xenarios, I., Hahn, W. C., Conaway, M., Carey, M. F., and Gioeli, D. (2010) CDK9 regulates AR promoter selectivity and cell growth through serine 81 phosphorylation. *Mol. Endocrinol.* **24,** 2267–2280
60. Zong, H., Chi, Y., Wang, Y., Yang, Y., Zhang, L., Chen, H., Jiang, J., Li, Z., Hong, Y., Wang, H., Yun, X., and Gu, J. (2007) Cyclin D3/CDK11p58 complex is involved in the repression of androgen receptor. *Mol. Cell. Biol.* **27,** 7125–7142
61. Ponguta, L. A., Gregory, C. W., French, F. S., and Wilson, E. M. (2008) Site-specific androgen receptor serine phosphorylation linked to epidermal growth factor-dependent growth of castration-recurrent prostate cancer. *J. Biol. Chem.* **283,** 20989–21001
62. Chymkowitch, P., Le May, N., Charneau, P., Compe, E., and Egly, J. M. (2011) The phosphorylation of the androgen receptor by TFIIH directs the ubiquitin/proteasome process. *EMBO J.* **30,** 468–479
63. Shu, S. K., Liu, Q., Coppola, D., and Cheng, J. Q. (2010) Phosphorylation and activation of androgen receptor by Aurora-A. *J. Biol. Chem.* **285,** 33045–33053
64. Steinkamp, M. P., O'Mahony, O. A., Brogley, M., Rehman, H., Lapensee, E. W., Dhanasekaran, S., Hofer, M. D., Kuefer, R., Chinnaiyan, A., Rubin, M. A., Pienta, K. J., and Robins, D. M. (2009) Treatment-dependent androgen receptor mutations in prostate cancer exploit multiple mechanisms to evade therapy. *Cancer Res.* **69,** 4434–4442
65. Robins, D. M. (2012) Androgen receptor gene polymorphisms and alterations in prostate cancer: of humanized mice and men. *Mol. Cell. Endocrinol.* **352,** 26–33
66. Jenster, G., van der Korput, H. A., Trapman, J., and Brinkmann, A. O. (1995) Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J. Biol. Chem.* **270,** 7341–7346
67. Cheon, C. K. (2011) Practical approach to steroid 5α-reductase type 2 deficiency. *Eur. J. Pediatr.* **170,** 1–8
68. MacLusky, N. J., and Naftolin, F. (1981) Sexual differentiation of the central nervous system. *Science* **211,** 1294–1302
69. Prince, F. P. (2001) The triphasic nature of Leydig cell development in humans, and comments on nomenclature. *J. Endocrinol.* **168,** 213–216
70. Reyes, F. I., Boroditsky, R. S., Winter, J. S., and Faiman, C. (1974) Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations. *J. Clin. Endocrinol. Metab.* **38,** 612–617
71. Sarkar, P., Bergman, K., Fisk, N. M., O'Connor, T. G., and Glover, V. (2007) Amniotic fluid testosterone: relationship with cortisol and gestational age. *Clin. Endocrinol.* **67,** 743–747